Construction, expression and functional evaluation of chimeric NKG2D receptor in murine NK cells

Undergraduate Research Thesis

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Abstract

Novel target development for immune-based cancer therapies is an active area of clinical interest. NKG2D is an activating immune cell receptor highly expressed on the surface of cytolytic natural killer (NK), NKT, and T-cells, making NKG2D an excellent target for immune therapy. Our lab developed a novel **bi**specific **k**iller-cell **e**ngager antibody (BiKE) targeting human NKG2D and CS1 receptors highly expressed in multiple myeloma (MM). BiKE binds to human NKG2D receptors, activating lymphocyte effector cells while binding and lysing CS1-expressing MM tumors (Fig. 1). BiKE does not bind murine NKG2D, so human lymphocytes and activating cytokines are injected to test BiKE in our orthotopic immune deficient mouse model. However, exogenous cytokines, such as IL-2 and IL-15, can also activate NK cell cytotoxicity, making it difficult to distinguish BiKE-mediated NK killing from cytokine-mediated killing. Our *in vitro* studies demonstrate that BiKE activates lymphocytes and lyses CS1-expressing tumors, but an immune competent mouse model with NK, NKT and T-cells expressing human NKG2D is needed to reliably assess BiKE efficacy *in vivo*.

In humans, NKG2D triggers NK cell activation by associating with adaptor protein DAP10, initiating cell cytotoxicity and IFN-γ release. In mice, NKG2D signals through DAP10 and DAP12, controlling cell cytotoxicity and IFN-γ release, respectively. The murine NKG2D transmembrane domain conveys adaptor protein specificity, allowing association with both DAP10 and DAP12. We have shown that substituting human NKG2D for mouse NKG2D does not elicit activation signals, likely due to DAP10/12 differences between species. Consequently, we created a chimeric NKG2D construct to evaluate BiKE efficacy in murine NK cells. **Overall, we hypothesize that a chimeric NKG2D construct with a human NKG2D ectodomain and mouse NKG2D transmembrane and intracellular domain will bind BiKE, initiating both cell cytotoxicity and IFN-γ release similar to human NKG2D.**

After creating our chimeric protein vector, we expressed it in both a murine liver NK (LNK) cell line and primary murine NK cells to test cell cytotoxicity. Our results in the LNK cell line did not show specific lysis of MM tumor cells, but the primary murine NK cells did show significant MM lysis ($p=0.0039$). During our testing, we determined an error in the protein binding domains of our chimeric protein and redesigned it. Using our new chimeric vector, we transiently expressed chimeric NKG2D in 293T cells, showing binding with anti-human NKG2D antibodies and BiKE. In this study, we show that a chimeric NKG2D receptor can bind human NKG2D ligands and initiate a cytotoxic tumor lysis response. While there is still testing to be done, we are confident moving forward that our redesigned chimeric NKG2D receptor will show functionality with BiKE and can be used to design our mouse model.

Introduction

Multiple Myeloma Cancer: An Overview

Multiple Myeloma (MM) is a cancer of the plasma cells, or differentiated B cells in the bone marrow (1). Typically B cells are only stimulated to form plasma cells when the immune system is fighting an infection, as plasma cells produce antibodies to neutralize and target foreign antigens (2). However, in MM this process becomes dysregulated and plasma cells begin overcrowding the rest of the bone marrow and producing high levels of M protein, a non-specific antibody (2). The high levels of M protein in the blood stream make the blood thicker, placing stress on the kidneys that, coupled with the high levels of calcium also characteristic of MM, leads to renal failure (3). In addition, the plasma cells crowd out other cells in the bone marrow critical to bone marrow function, including red blood cell precursors, white blood cell precursors, and platelets, leading to anemia, increased susceptibility to infection, decreased clotting ability, and a disrupted bone marrow microenvironment (2). Plasma cells also secrete osteoclast-activating factors (OAF) that activate osteoclasts in the bone marrow to break down bone tissue faster than the bone can be reformed, resulting in weak and brittle bones in many MM patients (4). Combined together, these symptoms prove to be formidable to treat and physicians often face challenges balancing current treatments with renal failure, anemia, and brittle bones.

MM accounts for nearly 10% of all hematological cancers, but the five-year survival rate is only 49.6%. While MM is a rare cancer, in 2017 over 30,000 people were diagnosed with MM and nearly 13,000 MM deaths were recorded. Median diagnosis age for MM is 69, with African-American men having the highest risk for developing MM and mortality (5). Unlike other cancers, patients are never considered cured and recurrent multiple myeloma

poses a significant clinical challenge for physicians due to damage to the kidneys and other organs as well as aggressive MM tumors (6). In light of these findings, MM has been a significant focus of clinical and benchtop research to address the unique needs of MM patients.

Current Multiple Myeloma Treatments

Once diagnosed with MM, most patients will undergo autologous stem cell transplant (ASCT). Hematopoietic stem cells are collected from patient bone marrow and then chemotherapy is used to kill all cells in the bone marrow, both malignant and normal cells. The healthy stem cells are then reintroduced to the patient to regenerate the blood cells in the bone marrow. While this treatment has a low mortality rate, most patients will relapse within two years because many cancerous stem cells are reintroduced to the patient during this process. Recent studies have shown that undergoing two ACST treatments six to twelve months apart can reduce the rate of relapse for patients, and Attal et. al. showed that the seven-year event free rate for patients with double ACST (20%) doubled from the control singular ASCT treatment (10%). However, this treatment has shown to be riskier for patients with the potential for more side effects and cancer relapse and, as a result, is not widely utilized (7).

If ACST transplant is not a viable option, allogeneic stem cell transplantation (allo-SCT) is considered. In this treatment, a matched donor donates their bone marrow to a MM patient. This approach is attractive because currently this is the only therapy that can cure MM. The tissue graft replaces cancerous plasma cells in the patient and studies have found that Graft Versus Host Disease (GVHD), a common side effect to tissue transplants when the

body rejects a tissue graft, can actually lead to the better overall survival of MM patients by mounting an immune response in the bone marrow. However, there is a high rate of treatment-related mortality, which discourages many doctors and patients from using this treatment plan. As a result, this treatment is only recommended for “ultra-high risk” MM, typically patients with genetic abnormalities that put them at risk for aggressive MM tumors (8).

In addition to traditional tissue grafts and chemotherapy, research during the past fifteen years has discovered several promising drugs that, when used in combination with other therapy techniques, hold promise for MM patients. One such drug is bortezomib, the first FDA-approved proteasome inhibition drug for cancer therapy. Unlike other chemotherapy drugs, bortezomib functions by reversibly inhibiting the 26S proteasome in the cell which disrupts protein degradation in the cell critical for maintaining cell cycle control; buildup of these cell cycle control proteins lead to apoptosis. Since MM tumors already have dysregulated cell cycle signal proteins, they are particularly susceptible to bortezomib. Bortezomib also has MM specific mechanisms, including preventing the breakdown of inhibitory kappa B which stabilizes the nuclear factor kappa B(NFkB) complex and prevent its further downstream signaling in MM cells. NFkB has been shown to promote MM tumor growth and decrease tumor cell dependence on the bone marrow microenvironment, making inhibiting NFkB signaling a clinically relevant treatment option (9). Treatment with bortezomib also decreases tumor cell adhesion, leading to apoptosis, and disrupts survival cytokine signals through IL-6, IGF-1, VEGF, and TNF-α. In addition to all these benefits, bortezomib inhibits angiogenesis, DNA repair, and abnormal osteoclast activity.

Bortezomib has been shown to be clinically effective when combined with the current chemotherapy drug dexamethasone, improving patient response rates up to 62% (10). Many studies indicate that bortezomib increases natural killer (NK) cell activity in MM tumor rejection by increasing MM tumor expression of MHC class 1 activating ligands and sensitizing the tumors to NK-mediated lysis, which will be discussed in the next section (11). Relapsed or non-transplant eligible MM patients can also take advantage of bortezomib, but patients suffering from renal failure may have to stop treatment due to renal complications. While it shows promise, bortezomib is currently only approved for recurrent MM patients or those ineligible for transplantation, so further drugs are needed on the market to address this need (10).

Natural Killer Cells

Natural Killer (NK) cells are a critical part of the body's immune response to detect and destroy infections. Unlike the T and B cells of the adaptive immune system, NK cells are large granular lymphocytes in the innate immune system, meaning that they do not adapt to new foreign antigens and they have a larger range of activation signals than cells in the adaptive immune system. NK cells continuously patrol the body until they encounter a source of infection, where a wide variety of activating receptors on the cell surface designed to detect large classes of foreign invaders are engaged. Lymphocytes of the adaptive immune system typically have restricted targets due to the unique activating receptors found on their cellular surface, such as with T cells, but NK cells are able to identify a wide range of stress ligands induced on host cells due to infection by foreign microbes, including major histocompatibility complex (MHC) class-1-chain-related–proteins (MICA & MICB) and

cytomegalovirus UL16-binding proteins (ULBPs) in humans and histocompatibility antigen 60 (H60), retinoic acid earl inducible cDNA clone-1 (Rae-1), and murine UL16-binding protein-like transcript 1 (MULT1) in mice. NK cells then respond to microbes similarly to T cells, causing target cell lysis and cytokine secretions to attract other lymphocytes to the infection site. Most notably, NK cells secrete IFNγ, a cytokine which, in addition to activating other NK cells, also helps mount a T cell immune response, activates antigen-presenting cells to upregulate MHC class ligands, activates macrophage-mediated cell killing, and prevents the proliferation of infected host cells (12). Both the wide-sweeping antigen recognition and cytotoxic response to infection make NK cells a key player in the immune system.

In humans, NK cells are typically defined by the expression of CD56, a 140 kDa neural cell adhesion molecule, and the absence of CD3, a cluster of differentiation 3 receptor expressed exclusively on T-cells. NK cells are hypothesized to develop in the lymph nodes and tonsils, allowing ready access to the circulation system, and are relatively short lived when compared to other granular lymphocytes. There are two major classes of NK cell development, $CD56^{bright}$ (cells with high expression of CD56) and $CD56^{dim}$ NK cells (cells with low expression of CD56). $CD56^{bright}$ cells are hypothesized to be the earlier stage of NK cell development in the secondary lymphocyte tissues with an increased capacity to secrete IFNγ. $CD56^{bright}$ cells mature into $CD56^{dim}$ cells with increased cytolytic capacity in the circulation system. NK cells have several pathways to induce a cytolytic response, mediated by NKG2D, NCR, NKp80, and nectin and nectin-like receptors. NK cells also have many inhibitory pathways to balance NK cell activity, including killer immunoglobulin-like receptors (KIRs) which recognize MHC class ligands or ‘self-molecules’ only expressed on host cells to distinguish host cells from foreign microbes and prevent NK cells from killing uninfected

host cells(13). The powerful cytolytic response activity and simple activation pathways have made NK cells a topic of intensive research as a critical piece of the immune system and as an attractive clinical target for immunotherapy.

Immune Evasion

Cancerous plasma cells (PC) express adhesion molecules that aid their binding with the bone marrow stromal cells (BMSC) in the marrow microenvironment. This adhesion activates IL-6 production and secretion in BMSC cells, which activates a paracrine IL-6 pathway leading to PC transcription and secretion of IL-6 which continues signaling to PC cells in an autocrine fashion. Tumor necrosis factor alpha (TNF-α) also contributes to this signaling cycle; by binding to BMSC cells, it also signals for IL-6 secretion and the expression of adhesion molecules. High levels of IL-6, in turn, decrease the number of regulatory T cells (T_{reg}) while increasing the number of IL-17 producing T cells (Th17). This increase in IL-17 increases osteoclast activity and leads to the further development of lytic bone disease. MM tumor cells also express an IL-17 receptor, causing the Th17 pro-inflammatory response to further fuel tumor growth and development. Th17 cells also inhibit the actions of Th1 T-cells, which typically support cytotoxic CD8+ T cells in rejecting cancerous cells (14,15). With decreased Th1 activity, MM prevents tumor surveillance and suppressing the body's immune response (14).

NK and NKT cells have also been implicated in MM tumor progression; studies have documented that NK cell differentiation is impaired in addition to functionality, primarily as a decrease of IFNγ production even in the presence of activating NK cell ligands. However, studies have documented that this response can be overcome, particularly by injecting

priming dendritic cells expressing α-GalCer, an activating NKT ligand (16). IL-6 decreases NK cell cytotoxicity as does transforming growth factor-beta (TGF-β), another prominent immune system regulator expressed by MM. TGF-β decreases the number of cytolytic granules in NK cells, inhibiting NK cell functionality via the CD16 receptor activating and antibody dependent cellular cytotoxicity (ADCC) pathways. The immunoglobulins produced by the MM plasma cells also decrease cytolytic granules, but research has shown that this effect can be reversed via IL-2 signaling (17). IL-15 is another cytokine important to the activation of NK cells, but, much like IL-17 mentioned above, MM cells express IL-15 receptors and use these signals to fuel their own tumor growth. Like many cancers, MM has been shown to shed ligands for NKG2D and other activating receptor on NK cells, leading to a downregulation in these receptors; this pathway will be discussed in more detail under the NKG2D heading (18). These results show that, while MM has manipulated the tumor microenvironment to inhibit immune surveillance and even uses cytokines to fuel tumor growth, the immune system can be retargeted to clear tumor cells.

Immunotherapy and Natural Killer Cell Multiple Myeloma Therapy

MM research in the last decade has primarily focused on redirecting the patient's immune system to attack MM tumors. The recent wealth of knowledge in the field of immunology has guided translational immunotherapy cancer research. Researchers are attempting to redirect or reactivate the body's natural immune defenses by targeting immune cells to directly attack cancer tumors. By specifically targeting tumor cells with the immune system, researchers aim to reduce the off-target side effects of common chemotherapy drugs and lead to specific apoptosis of cancerous cells. Combined with

currently available cancer treatment techniques, these therapies hold much promise and have been areas of intensive clinical and research interest.

Perhaps the most successful class of immunotherapy drugs is the chimeric antigen receptor (CAR) used with T cells. Initially, CAR T cells were used to put acute lymphoblastic leukemia patients into complete remission, but this therapy has expanded into MM treatment. After initial chemotherapy and ASCT, a patient's T cells are isolated and transduced to express a CD19-specific chimeric antigen that targets MM tumors. While CD19 is reported to be expressed infrequently on MM cells, studies show that some drug-resistant strains of MM express CD19, making these receptors an excellent target for CAR therapy. Using the CAR design, the receptor contains an intracellular T cell activation domain, a costimulatory domain, and an antigen recognition domain, in this case specific to CD19. These T cells then specifically target MM cells expressing CD19 ligands, initiate cell cytotoxity and IFNγ release, and lead to complete cancer remission (19). Similar projects have focused on a B-cell maturation antigen target for MM, but these clinical trials did not show the same level of mounting immune response as the CD19 CAR trial (20).

One disadvantage to the CD19 CAR therapy is that studies have found over 95% of MM patients do not CD19 expression on MM tumor cells, meaning many patients cannot take advantage of this treatment (21). In addition, this therapy places patients at risk for cytokine-release syndrome (CRS), which occurs when large numbers of the immune system effector cells are stimulated simultaneously and begin releasing activating cytokines, leading to further activation of the immune system and severe symptoms for patients. Patients with CRS can have fever, nausea, chills, hypotension, kidney dysfunction, respiratory arrest, and

Graft vs Host Disease, and some cases have even ended in patient mortality (21,22). While steroids and blocking antibodies can be administered to reduce the symptoms and severity, CRS remains a critical concern of immunotherapy and in particular the CD19 CAR clinical trials. In most cases these symptoms are manageable, but some patients may experience life-threatening symptoms, making this a leading cause of concern in immunotherapy (23).

While CAR T cell therapies have shown strong potential, CAR NK cell therapies have been largely unexplored and pose a solution to many of the side effects of CAR T cell therapies. Our lab has previously created and tested a CS1-specific MM CAR NK cell therapy *in vivo* and *in vitro*. CS1 is a receptor specifically and highly expressed on the surface of MM tumor cells with low expression on B, NK, and T cells. Clinical studies have also found that CS1 remains highly expressed on MM patients even in remission, making it an excellent target for MM treatment. While CS1 function in normal plasma cells is unknown, in cancerous MM cells is hypothesized to promote cell adhesion, the growth of tumors, and interactions with BMSCs (21,24). This CS1 CAR therapy demonstrated specific MM cell lysis and proves to be a promising direction for clinical trials. In addition, NK cells have the added advantage of decreasing CRS, tumor lysis syndrome, graft versus host disease, and off-tumor effects; NK cells do not exhibit clonal expansion in vivo like T cells, limiting the response of NK cells to prevent side effects (21). NK cells also do not need to be primed as part of the innate immune system, meaning that activating cytokines may not need to be injected as part of CAR NK cell therapy which could prevent NK cells from becoming overactive and causing patient side effects. Combined with previous reports that NK cell activity is impaired in the progression of MM, this therapy shows promise at redirecting NK cells to reject cancer tumors but has yet to pass clinical trials or be approved by the FDA.

Finally, **bi**specific **T**-cell **e**ngager proteins (BiTE) have shown significant potential in retargeting immune effector cells to attack cancer tumors. This molecule consists of two single-chain variable antibody fragments connected by a linker. One of these chains will target an activating receptor on an immune effector cell, such as T cells, and the other chain will target a marker or receptor on cancerous cells. By linking together the single-chain variable fragments of two different antibodies, BiTE can activate cytotoxic T-cells, specifically bring them into direct contact with cancerous cells, and initiate tumor cell lysis. The most successful bispecific antibody fusion protein treatment is the bispecific T-cell engager (BiTE) protein produced by Amgen, which is the first BiTE to be approved by the FDA. Designed for the treatment of B cell leukemia, it connects CD3 and CD19 receptors on T-cells with specific cancer targets, but this treatment only activates T-cells to reject cancer (25). The modular design of BiTE allows drug companies and researchers to change out the antibody fragments to target different receptors and markers, making BiTE proteins the focus of intensive clinical cancer research as a potential therapeutic drug.

While BiTE molecules activate T-cells, some researchers are creating **bi**specific **k**iller-cell **e**ngager proteins (BiKE), using the same modular design of BiTE but targeting NK cell activation instead of T-cells. Various BiKE proteins have shown to be effective at rejecting cancer

Figure 1. BiKE Mechanism

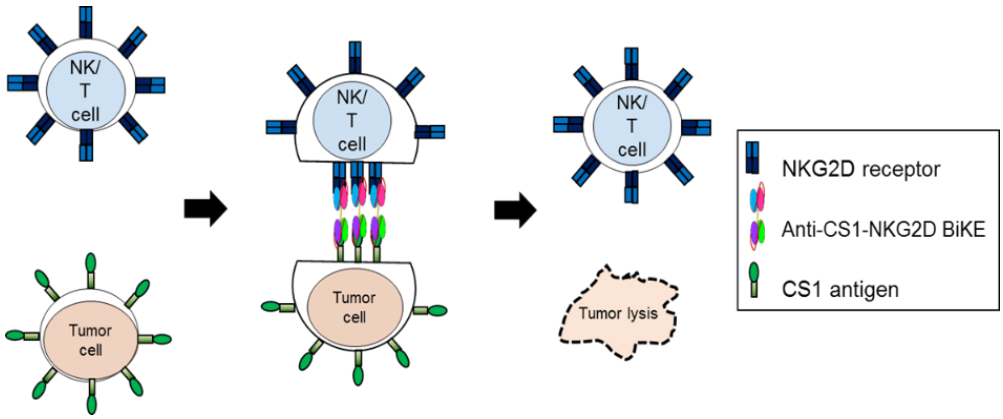

Figure 1. BiKE specifically binds to NK, NKT and T cells and CS1-expressing tumor cells, forming a link between the two cells, activating NK/T cells, and lysing tumor cells.

tumors in many different cancer lines, but few have passed through clinical trials. One BiKE has successfully been tested in the treatment of acute myeloid leukemia (AML) using the single-chain variable fragment for the activating CD16 receptor on NK cells and the single-chain variable fragment for the AML antigen CD33. This BiKE therapy was shown to effectively redirect NK cell activity that had been suppressed in AML patients and led to tumor cell lysis regardless of disease stage, but the drug has yet to proceed to clinical trials (26). The advantage of BiKE over other immunotherapy treatments is its off-the-shelf therapy design; BiKE does not need to be modified for individual patients, thus making the treatment quicker, cheaper, and readily available for a wider range of cancer patients. In addition, the modular design of BiKE simplifies the drug creation process and makes it easy to modify BiKE as new cancer targets and activating receptors are discovered (27). Our lab has focused on the potential of BiKE in MM therapy and how to take advantage of a wide range of immune effector cells in our drug design (Fig. 1).

Multiple Myeloma BiKE Treatment

Our lab has created a BiKE therapy using an anti-CS1 and anti-NKG2D single-chain variable antibody fragments connected via a linker to direct the immune system to attack MM cancer cells (Fig. 2). As mentioned above, CS1 is a glycoprotein specifically expressed on the surface of MM tumor cells (28). NKG2D is an immune system activating receptor found on the surface of three types of killer cells, NK, T, and NKT cells (13). BiKE

Figure 2. BiKE Structure

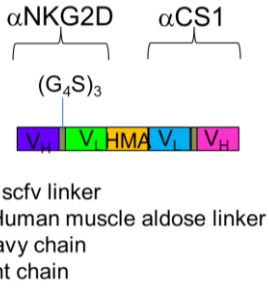

Figure 2. BiKE is composed of the single-chain variable fragment of anti-NKG2D and anti-CS1 antibodies connected by a flexible linker.

physically brings together several types of immune effector lymphocytes into direct contact with MM tumor cells while simultaneously activating tumor cell lysis and IFNγ release (Fig. 3). IFNγ is a cytokine released by effector lymphocytes to recruit and activate nearby immune effector cells, creating a second wave of MM cancer cell lysis. Unlike other BiKE or CAR therapies, our BiKE antigen would activate a wide range of immune effector cells, such as NK, NKT, and CD8(+) αβ and γδ T-cells, generating a larger tumor rejection response while simultaneously limiting CRS side-effects; due to the 57 kDa size, BiKE can be filtered out of the bloodstream and limit long-term activating immunotherapy side effects characteristic of other immunotherapies. Studies show that the kidneys filter plasma proteins less than 60 kDa out of plasma, allowing BiKE to filter out of the bloodstream (29). These concerted efforts would overcome the suppressive tumor microenvironment and lead to MM cell lysis and tumor rejection while reducing patient safety concerns.

Figure 3. BiKE Tumor Lysis

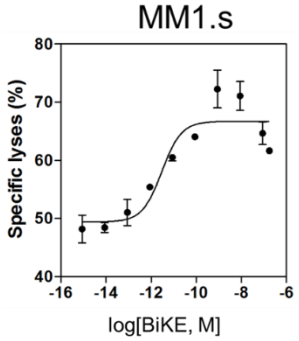

Figure 3. Human NK cells specifically lyse a CS1-expressing MM cell line when cocultured with BiKE.

Our *in vitro* studies show that BiKE activates the NKG2D receptor on NK, NKT, and CD8(+) αβ and γδ T-cells while putting these effector cells in direct contact with MM via the CS1 receptor. This interaction leads to the specific lysis of CS1-expressing tumor cells and the production of IFNγ. Taken together, this data suggests that BiKE would be an effective anti-neoplastic drug against MM *in vivo* by uniquely engaging three different types of lymphocyte effector cells to reject MM tumors. To the best of our knowledge, no other immunotherapy drug has attempted to target multiple subsets of lymphocyte effector cells,

setting our BiKE apart from other drugs and potentially providing patients with another effective treatment for MM.

In Vivo BiKE Testing

While our *in vitro* data shows that BiKE would be an effective drug, our *in vivo* modeling system presents several confounding variables that cloud the quality of our data. Human NKG2D, the activating receptor for NK cells in BiKE, has both signaling and biochemical dissimilarities between humans and mice, making our human BiKE protein unable to bind with murine NKG2D. As a result, to test our compound we must inject human NK cells into our mouse model so we can observe the tumor rejecting effects between BiKE and human NK cells. Our current model irradiates NSG mice to destroy the endogenous immune system and then injects MM tumor cells (MM1.s cell line) into the mice. On day seven, the mice are then injected with human NK cells isolated from PBMC. Following this, each day the mice are injected with BiKE, as well as human IL-2 to promote human NK cell survival signaling and human IL-15 for NK cell activation (17,30). Tumors are imaged on day fourteen and twenty-one, and we see that mice receiving the BiKE treatment have prolonged survival and tumor clearance than the control (Fig. 4). However, IL-2 has been shown to

independently activate NK cell cytotoxicity by upregulating the amount of NKG2D expressed on the surface of NK cells, so we are unable to attribute tumor cell clearance solely with the

Figure 4. BiKE *In Vivo* Testing

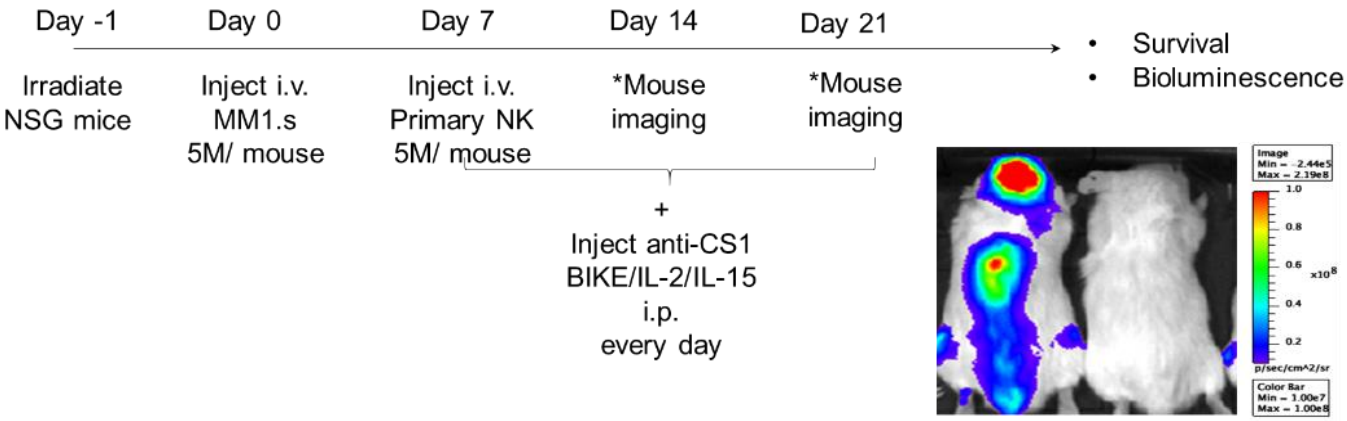

Figure 4. Timeline of murine BiKE testing. Irradiated mice are injected with MM cells and then injected with human NK cells, IL-2, IL-15, and either control BiKE or anti-CS1 BiKE daily for two weeks. On the left, tumor growth is shown using bioluminescent dye in a mice treated with control BiKE, while no tumor growth is seen on the mouse on the right receiving anti-CS1 BiKE treatment.

BiKE treatment (17). In addition, by injecting both human NK cells and BiKE into the mice, we are manipulating the murine immune system, introducing further variability into our experiment. To generate further preclinical data and potentially proceed to clinical trials, an immunocompetent mouse model system must be created to test our BiKE construct.

NKG2D Signaling Differences in Humans and Mice

NKG2D is a C type-like lectin single-pass transmembrane protein that forms homodimers in the plasma membrane. In humans, NKG2D noncovalently associates with four DNAX-activated protein of 10 kD (DAP10) subunits in the lipid bilayer through a critical aspartic acid residue and form salt bridges with the arginine residue in the transmembrane domain of NKG2D (31,32). Wu et. al. has shown that this NKG2D-DAP10 complex stably associates together in humans, and the expression of DAP10 is required to stabilize NKG2D

expression (33). NKG2D recognizes cell surface ligands upregulated by stress, typically from viral infection, tumorigenesis, and DNA damage (33). When NKG2D binds one of its activating ligands, including major histocompatibility complex (MHC) class 1-related ligands, stress-inducible surface glycoproteins MICA and MICB, and human cytomegalovirus UL16 binding proteins (ULBPs), DAP10 becomes phosphorylated and causes a signaling cascade (34,35). Because one NKG2D homodimer associates with four DAP10 protein subunits, the signal is immediately amplified and increases NKG2D signaling sensitivity (36). The phosphatidylinositol 3-kinase (P1-3K) becomes phosphorylated and activates AKT, leading to a phosphorylation cascade that activates both NK cell cytotoxicity and INFγ cytokine release (Fig. 5) (32). In humans, there is only one NKG2D signaling pathway that leads to the specific lysis of target cells and IFNγ release to activate other cytolytic effector cells and assist in tumor rejection.

Figure 5. NKG2D Signaling in Mice and Humans

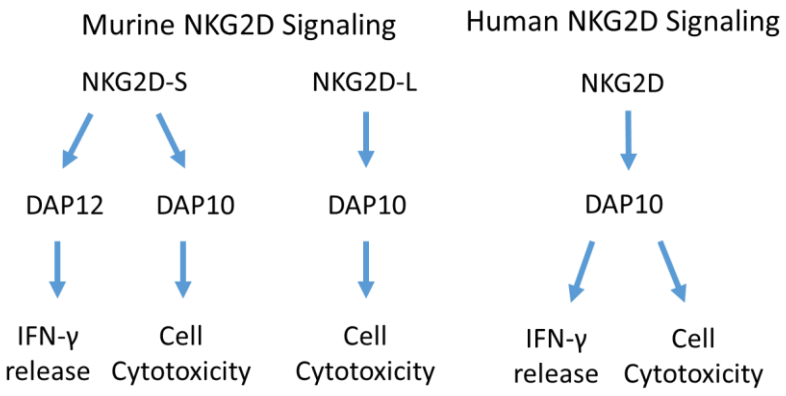

Figure 5. Differences between murine and human NKG2D signaling. In mice, two transcript variants of NKG2D control signaling through DAP10/12. In contrast, human NKG2D only has one transcript that controls NKG2D signaling using only DAP10.

In mice, there are two separate NKG2D signaling pathways with two alternatively spliced versions of NKG2D. The first protein, called NKG2D-long (NKG2D-L) works in a similar manner to humans; NKG2D-L signals through DAP10 and activates the PI3 kinase for

downstream signaling, but only cell cytotoxicity is activated. There is a second protein, called NKG2D-short (NKG2D-S), which is missing thirteen amino acids at the N terminus compared to NKG2D-L and is only translated when the NK cell has already been activated by other signaling pathways (Fig. 5)(12,37). Studies show that there is significant sequence divergence in human NKG2D and murine NKG2D transmembrane domains, allowing murine NKG2D to associate with both DAP10 and DAP12. When NKG2D-S pairs with DAP10, it still activates the NK cell cytotoxicity pathway. However, pairing with DAP12 initiates IFNγ release through the spleen tyrosine kinase (Syk) and NFκB signaling pathways (12,32,37). Using the NKG2D-S pathway, both cell cytotoxicity and IFNγ release can be initiated like the human signaling pathway, but it uses both DAP10 and DAP12 to accomplish both signaling pathways. Murine NKG2D also binds different ligands, many of which are homologs to human NKG2D signaling molecules, including retinoic acid-early (RAE-1) molecules, histocompatibility antigen 60 (H60), murine UL16-binding protein-like transcript 1 (MULT1), and major histone compatibility complex homologs (34,38). Because the extracellular binding domains of NKG2D bind different ligands in humans and mice, our human BiKE protein is unable to bind murine NKG2D and subsequently is affecting the quality of our *in vitro* testing data.

NKG2D Specific Tumor Evasion Methods

Tumor cells have developed many techniques to dampen the anti-tumor of effects of the NKG2D receptor. One such mechanism is by secreting tumor growth factor (TGF)-β, an anti-inflammatory cytokine. TGF-β deactivates NKG2D signaling in a dominant fashion, overriding the presence of NKG2D ligands and upregulating NKG2D cytokines, such as IL-2

and IL-15, which stabilize DAP10 expression levels by promoting glycosylation (17,39). It has been shown that TGF-β downregulated NKG2D expression on cytotoxic lymphocytes by decreasing DAP10 transcription via interfering with the RNA Polymerase II association with the DAP10 promoter (17,39). It has also been shown that TGF-β disrupts the ratio of $CD16^{dim}$ and $CD16^{bright}$ NK cells, skewing the overall NK cell population to $CD16^{dim}$ NK cells with low expression levels of NKG2D (40). TGF-β constitutes a major pathway by which cancerous cells decrease NKG2D anti-tumor activity.

Another pathway by which cancer cells downregulate NKG2D receptor expression is by maintaining high levels of cell-surface bound NKG2D ligands. Studies have demonstrated that some cancer patients with tumors expressing high levels of NKG2D ligands had NK cell populations with decreased NKG2D expression, specifically in thymomas patients with high NKG2D ligand expression (41,42). Mouse studies have also confirmed this observation; mice that constitutively expressed MICA and RAE-I ligands had NK cell populations that were unable to reject RAE-I expressing tumors (41). Researchers have proposed that, while initially NKG2D ligand-expressing tumors may easily be rejected by NK cells, constitutive expression of these ligands leads to a knockdown of NKG2D expression, internalization of NKG2D receptors on cytotoxic lymphocytes, or dampens the NKG2D signaling pathway and response (39,41). As a result, tumors with continually high NKG2D ligand expression pose a clinical challenge to oncologists and their patients.

The discovery that tumor cells often shed NKG2D ligands, by either cleaving surface proteins or exocytosing alternatively-spliced soluble versions of the ligands, has further complicated researchers' model of NKG2D tumor cell evasion. Evidence shows that soluble

MICA secreted by tumors decrease NKG2D responsiveness, either by overstimulating the NKG2D pathway and leading to decreased expression or endocytosis of the receptor, or by occupying the NKG2D receptor site and preventing it from interacting with NKG2D ligands on the surface of tumor cells (43). Another study used a mouse line with constitutive expression of soluble MULT1 and tumors expressing Rae-1 ligands to test this hypothesis. Their results show that soluble MULT1 decreased NKG2D- mediated cell killing by causing endocytosis of NKG2D receptors and blocking NKG2D binding sites, but this activity was recovered when mice were treated with anti-MULT1 antibodies (12). Evidence shows that inflammatory cytokines can overcome this effect by increasing NKG2D expression, and we hypothesize that our BiKE will have a similar effect overcoming this type of immune evasion (43). However, NKG2D shed ligands are a recent discovery, and some reports have found that shed ligands increase NKG2D sensitivity by upregulating the expression of NKG2D and lead to better tumor rejection (44). In addition, it has also been reported that MM cells shed CS1-soluble ligands, but research has not shown if this has an effect on immune evasion or NKG2D surface expression (45). Further research must be conducted to gain a better understanding of the NKG2D shed ligand's role in immune evasion.

NKG2D Chimeric Protein Project

Previous studies indicate that human NKG2D may be able to initiate signaling in a murine NK cell due to 76% DAP10 sequence homology between species, conserved PI-3 kinase phosphorylation domains in DAP10 between species, and human NKG2D co-localization with both murine and human DAP10 (32). However, when our lab tested human

NKG2D signaling in murine NK cells neither IFN-γ release or cell cytotoxicity was observed (Fig. 6).

Figure 6. Human NKG2D Binding in Murine NK Cells

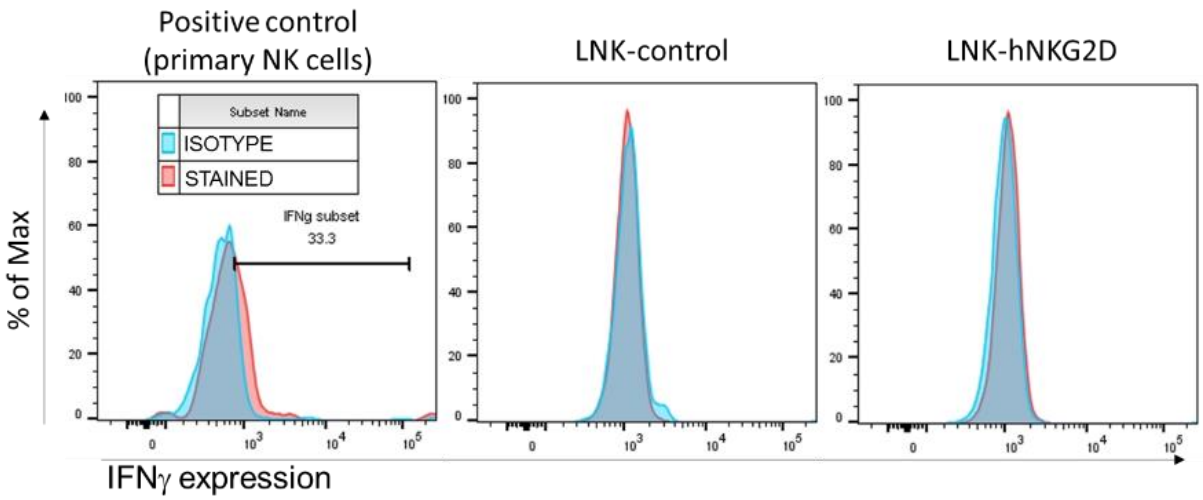

Figure 6. Human NKG2D expressed in mouse NK cells showed no IFN-γ release. Murine NK cells showing human NKG2D expression were stained with gold-standard anti-human NKG2D antibodies and crosslinked with goat anti-mouse antibodies. A golgi transport inhibitor was added to prevent IFN-γ exocytosis. Control and treatment groups showed no difference in IFN-γ levels.

In light of these experiments, we created a chimeric NKG2D protein that would bind to the human anti-NKG2D anti-CS1 BiKE protein and initiate an intracellular murine NKG2D response. The chimeric NKG2D construct has the extracellular domain of human NKG2D, the transmembrane domain of murine NKG2D-S, and the intracellular domain of murine NKG2D-S. This construct will bind the human BiKE protein, pair with both DAP10 and DAP12 in murine NK cells, and mount a murine immune response leading to both cell cytotoxicity and IFNγ release. Here, I report our findings on:

1. Chimeric NKG2D expression in murine NK cells;
2. Evaluation of BiKE binding to the NKG2D chimeric construct;

3. Evaluation of the function of BiKE binding to the NKG2D chimeric construct.

Results from these in vitro experiments will provide the data necessary to proceed towards creating a chimeric NKG2D knock-in mouse model and preclinical BiKE testing in vivo. To the best of our knowledge, this will be a novel mouse model and will further NKG2D research.

Methods

BiKE antibody construction, expression, and purification

Our BiKE was designed *in silico* using DNA from the mouse anti-human NKG2D and anti-human CS1 single chain variable fragment monoclonal antibodies joined by a non-immunogenic human aldose protein linker. A secretory H7 signal peptide was added to the N-terminus of the protein sequence, and a six-histidine tag was added to the c-terminus of the anti-CS1 single chain variable fragment. After codon optimization, the sequence was synthesized and subcloned into a pCDH lentiviral vector with a GFP reporter gene to characterize expression levels (SBI Bioscience, Palo Alto, CA, USA). A CHO-S cell line (Invitrogen, Waltham, MA, USA) was created by sorting for highly GFP expressing cells using BD Aria II (BD Biosciences, San Jose, CA, USA). A CDR3 deletion mutation was made to create a negative binding bispecific antibody control (control BIKE). The supernatant of CHO-S cultures was purified and concentrated using size-exclusion chromatography. Samples were sequentially dialyzed with PBS using 100 and 50 kDa pore filters. Purified BiKE was then analyzed by SDS-PAGE and stained with Coomassie Brilliant Blue for size estimation and quality control. Protein concentration was calculated using the NanoDrop 2000 (Thermo Ficher Scientific, Watham, MA, USA).

Multiple myeloma cell, murine natural killer cell, human natural killer cell, and 293T cell cultures

The multiple myeloma cell line MM.1S was obtained from ATCC (Manassas, VA, USA) and cultured under the manufacturer's instruction. A murine liver natural killer cell line (LNK) was obtained from Dr. Jianhua Yu and cultured in RPMI with 10% FBS, 5% sodium pyruvate, 0.1% BME, and 6.7 μL/mL IL-2. All cell lines were routinely checked for

mycoplasma and passaged for no more than two months. Human peripheral blood mononuclear cells (PBMCs) were isolated from the peripheral blood of healthy donors by Ficoll-Paque Plus (GE Healthcare Life Science) gradient density with the approval of the Institutional Review Board at The Ohio State University. Human NK cells were isolated from peripheral blood using the RosetteSep human NK isolation kit (Stem Cell Technology, Cambridge, MA, USA) following the manufacturer's instructions. Primary murine natural killer cells were isolated from the spleen of C57BL/6 sacrificed mice. Spleen were homogenized and filtered through 100um cell strainer. The NK Cell Isolation Kit II, a negative murine NK selection kit, was used to select for murine NK cells according to manufacturer instructions (Milenyi Biotec, Auburn, CA, USA). Murine NK spleen cells were cultured in RPMI with 10% FBS and 3.3 μL/mL IL-2. 293T cells were obtained from SBI Sciences and were cultured in DMEM medium with 10% FBS and confluency was maintained.

NKG2D vector design

mRNA from human and murine NK cells was isolated and the NKG2D gene was amplified using a cDNA reverse transcriptase kit, Vilo cDNA Synthesis Kit (ThermoFisher Invitrogen, Watham, MA, USA). From this DNA, three NKG2D viral vectors were made using a pCDH lentiviral vector: a control vector, a full length human NKG2D vector, and a chimeric NKG2D vector. The control vector simply had an empty pCDH vector with no NKG2D DNA introduced. The full length human NKG2D vector (NKG2D-full) used restriction sites to insert the full human NKG2D cDNA sequence into the pCDH vector. The third vector, the chimeric NKG2D vector (NKG2D-chim), was constructed to have an extracellular human NKG2D binding domain, a murine NKG2D-S transmembrane domain, and murine intracellular

signaling domain (Fig. 7). Restriction enzyme cut sties were inserted into the human and murine NKG2D sequences using PCR-directed mutagenesis to ligate the sequences together. The DNA for the vectors was subcloned into the pCDH vector. We redid our NKG2D-chim DNA sequence after finding errors in the protein binding domains created with misdesigned primers. This DNA sequence had the same structure as NKG2D-chim vector, but restriction sites were not inserted into the sequence and the DNA sequence was codon optimized for murine expression. NKG2D-chimR was synthesized by Quintarabio into a pCDH vector. All vectors were sequenced using The Ohio State University Comprehensive Cancer Center Sanger Sequencing services.

Figure 7. Chimeric NKG2D Receptor

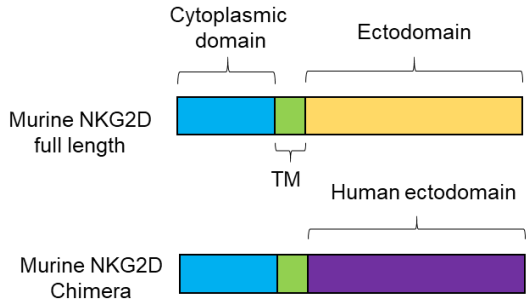

Figure 7. Design of the chimeric NKG2D receptor. cDNA for the murine NKG2D-S cytoplasmic and transmembrane was joined with cDNA for the human NKG2D ectodomain.

Next, the vectors were transfected into 293T cells using Lipofectamine according to the manufacturer's instructions. The pCDH vectors were co-transfected with helper plasmids psPax2, a packaging viral vector, and pHDMG, a viral envelope vector, in an Opti-MEM medium into 293T cell cultures. Sixteen hours after transfection, the Opti-MEM medium was exchanged for DMEM medium. Twenty-four hours later, the supernatant of the transfected 293T cells was collected and filtered for cellular debris through a 0.45µm filter, and the viral particles were concentrated using an ultra-centrifuge and a sucrose cushion at 25,000 RPM for 2 hours at 4°C. The virus is then resuspended in X-Vivo 15 medium and a viral titer was performed to measure the concentration of virus. For the titering experiment,

293T cells are again used and the virus is serially diluted. Concentration is measured by GFP expression of 293T cells using a BD LSRII flow cytometer. Viral stock is then stored at -80°C.

Viral transduction of murine natural killer cells

Murine NK cells are transduced with virus in a 96-well plate. After twenty-four hours, the NK cells undergo a second round of transduction. NK cells are then harvested 24 hours later and suspended in RPMI medium with 10% FBS, 5% sodium pyruvate, 0.1% BME, and 6.6 μL/mL of IL-2. After three days, NK cells were checked for vector GFP expression and sorted using a BD LSRII flow cytometer.

Murine natural killer liver cells and BiKE cytotoxicity assays

Murine LNK cells transduced with control and NKG2D-chim vectors were plated in a 20:1 ratio with MM.1S tumor cells. Cells were either treated with PBS, 10 μg/100 μL of control BiKE, or 10 μg/100 μL BiKE in 10% FBS and 1% antibiotic RPMI medium in a flow-based cytotoxicity assay. LNK NKG2D-chim cells incubated with PBS or control BiKE were used as a negative control. The plate was incubated for 24 hours. During flow cytometry sorting, NK cells were identified as GFP positive due to the expression of the GFP gene in the pCDH lentiviral vector while MM.1S cells were stained for human CD45 and sytox to determine the percentage of tumor clearance.

Primary murine natural killer spleen cells and BiKE cytotoxicity assays

Murine spleen cells transduced with control, NKG2D-full, and NKG2D-chim vectors were plated in a 20:1 ratio with MM.1S tumor cells and 10 μg/100 μL BiKE in 10% FBS and 1% antibiotic RPMI medium in a flow-based cytotoxicity assay. Multiple myeloma cells

incubated with BiKE were used as a negative control. The plate was incubated for 24 hours. During flow cytometry sorting, NK cells were identified as GFP positive while MM.1S cells were stained for human CD45 and sytox to determine the percentage of tumor clearance.

293T cell binding assay for BiKE

293T cells were transformed with the control vector and the NKG2D-chimR vector using lipofectamine according to manufacturer instructions. After 16 hours the cells were checked for transient expression of NKG2D. Cells were washed with MACS buffer, and both the control and the NKG2D-chimR cells there were four staining groups: isotype APC, human anti-NKG2D, control BiKE conjugated with APC, and BiKE conjugated with APC. After incubation in the dark for twenty minutes at room temperature, the cells were washed with MACS buffer and then checked for NKG2D binding via flow cytometry.

Results

pCDH NKG2D-full and NKG2D-chim vector subcloning

To create a vector expressing the full length human NKG2D cDNA, PCR was performed with primers introducing restriction enzyme sites for ligation into the pCDH vector. An EcoR1 site was added at the 5' end of the amplified gene, while a Not1 restriction enzyme was added at the 3' end of the gene. PCR showed successful amplification of the 650 base pair gene (Fig. 8), and the fragment was then digested by restriction enzymes and then ligated into the pCDH vector with T4 DNA ligase. LNK and murine NK spleen cells were transduced with the vector, and GFP expression confirmed plasmid integration and gene expression during transduction experiments (Fig. 11).

Figure 8. Full Length Human NKG2D PCR

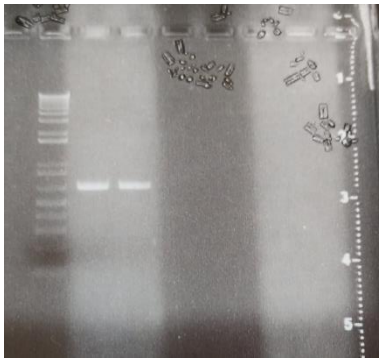

Figure 8. Full length NKG2D PCR from healthy NK donor cells. Both lanes show PCR products from two different donors, with a PCR product around 650 base pairs.

Figure 9. Chimeric NKG2D PCR

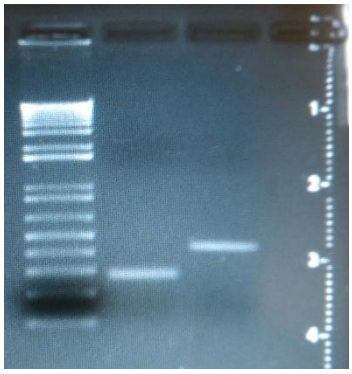

Figure 9. PCR amplification of the murine NKG2D intracellular and transmembrane domains (Lane 1) and the human NKG2D ectodomain (Lane 2). The murine NKG2D fragment is approximately 250 base pairs and the human NKG2D fragment is approximately 450 base pairs.

The chimeric NKG2D vector was subcloned into pCDH in a similar manner. Both murine NKG2D-S and human NKG2D cDNA was amplified using PCR primers that inserted restriction enzymes into the sequence for subcloning. For the murine intracellular and transmembrane domains, an EcoR1 cut site was introduced on the 5' end of the sequence, and a BamH1 site was introduced at the 3' end. The human NKG2D extracellular domain was amplified with BamH1 at the 5' end and Not1 at the 3' end. PCR showed successful amplification of a 252 base pair fragment of murine NKG2D-S cDNA and a 435

base pair fragment of human NKG2D (Fig. 9). Each fragment was digested with their respective restriction enzymes and ligated into a pCDH vector. LNK and murine NK spleen cells were transduced with the NKG2D-chim vector, and GFP expression confirmed plasmid integration and gene expression during transduction experiments (Fig. 11). High GFP expression was correlated with high protein construct expression (either human NKG2D or chimeric), so we sorted for GFP expression. We did not sort with anti-human NKG2D antibodies because they prematurely activate the murine NK cells and can alter the results of our functionality assays.

The NKG2D-chimR vector was sent for sequencing, so only GFP expression had to be confirmed during transduction to show gene expression (Fig. 11).

Murine natural killer liver cells and BiKE cytotoxicity assays

Figure 10. Chimeric NKG2D LNK Cell Cytotoxicity Assay

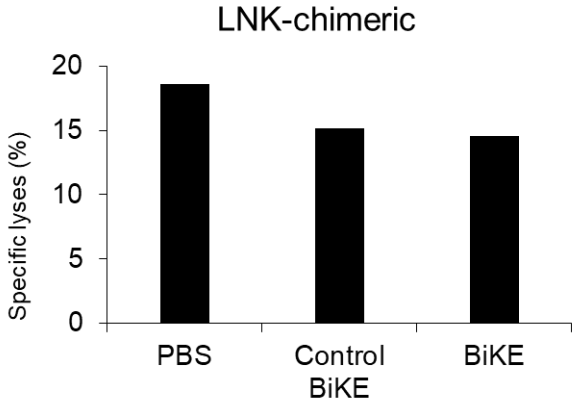

Figure 10. LNK chimeric cells were seeded in a 20:1 ratio with MM cells and treated with PBS, control BiKE, and BiKE to test the specific lysis of MM cells.

LNK NKG2D-chim cells were incubated with PBS, control BiKE, and BiKE in the presence of MM cells and tested for tumor clearance. LNK NKG2D-chim cells were found to have lower MM tumor clearance, but these results were not statistically significant ($p=0.1$)

(Fig. 10). This indicates that the chimeric receptor is not functioning properly in LNK cells. These results are representative of other LNK chimeric receptor cell cytotoxicity assays.

Primary murine natural killer spleen cells and BiKE cytotoxicity assays

Murine NK spleen cells were transduced with the empty pCDH vector, NKG2D-full vector, and NKG2D-chim vector plasmids (Fig. 11). Cells were analyzed with flow cytometry to check the percentage of transduced cells indicated by the GFP expression. The cells were then subsequently sorted using BD Aria II cell sorter to sort all transduced cells. These cells were used for subsequent cytotoxicity assays

Figure 11. Primary Murine Spleen Cell Transduction

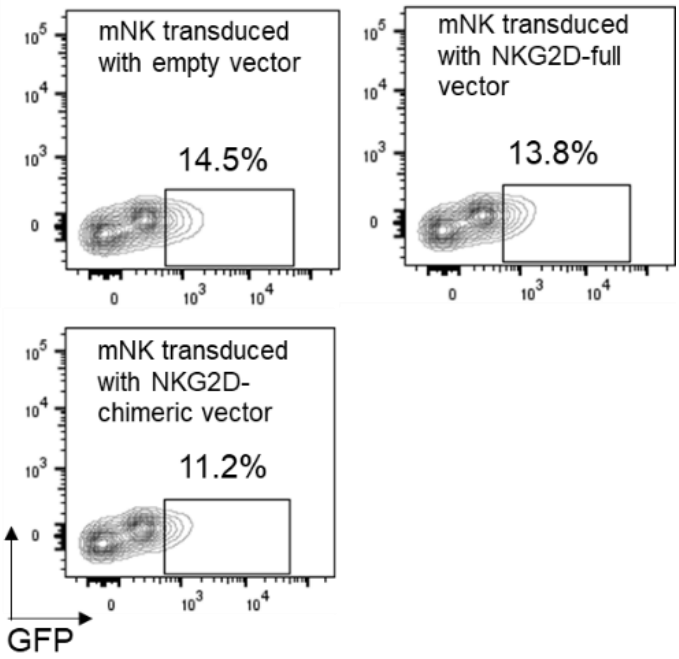

Figure 11. Transduced murine NK cells were sorted for empty vector, NKG2D-full, and NKG2D-chimR GFP expression using flow cytometry.

After incubating the murine NK cell control, NKG2D-full, and NKG2D-chim with MM cells and BiKE for 24 hours we found that the NKG2D-chim cell lines exhibited statistically

significant tumor clearance (more than 12%) than the NKG2D-full and control cell lines (p=0.0039) (Fig. 12).

Figure 12. Primary Murine Spleen Cell Transduction

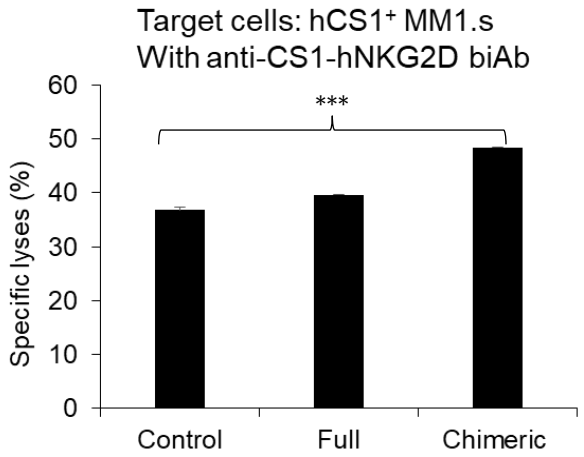

Figure 12. Murine NK cells transduced with an empty pCDH vector, NKG2D-full, and NKG2D-chim were incubated with BiKE and MM cells for 24 hours. Percentage of tumor clearance was observed.

Transmembrane domain comparison between NKG2D-chim and redesigned NKG2D-chim R vector

Upon comparing our sequence data between our NKG2D-chim vector and our desired chimeric receptor (later called NKG2D-chimR), we found two errors in the DNA sequence. As shown in Figure 13, a segment of the murine extracellular domain was added to the sequence in between the murine transmembrane domain and the BamH1 cut site used to create the vector. In addition, part of the human extracellular domain was absent from the sequence in the NKG2D-chim vector, as shown in Figure 14. The new vector, NKG2D-chimR, was designed to contain only the murine NKG2D-S transmembrane domain and the human NKG2D extracellular domain as originally intended. The NKG2D-chimR vector was constructed at Quantarabio to correct the sequence and eliminate the restriction enzyme cut sites.

Figure 13. DNA Comparison between NKG2D-chim and NKG2D-chimR Vectors

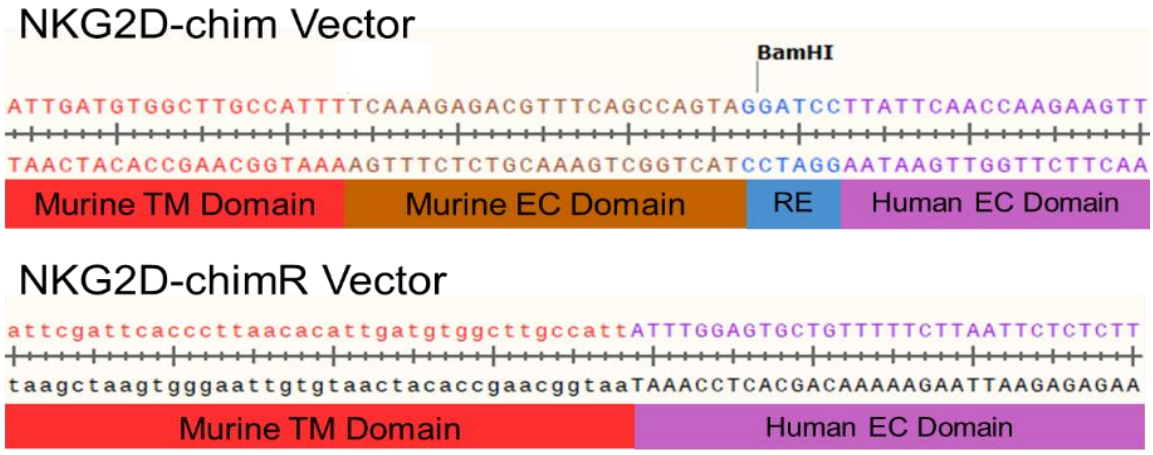

Figure 13. The DNA sequence for the NKG2D-chim receptor is compared with the NKG2D-chimR sequence. The NKG2D-chim vector correctly contains a murine NKG2D-S transmembrane domain, a BamH1 restriction enzyme cut site, and a human NKG2D extracellular domain. However, when compared with the NKG2D-chimR sequence, we see that NKG2D-chim sequence incorrectly contains a murine extracellular domain.

Figure 14. Protein Comparison between NKG2D-chim and NKG2D-chimR Vectors

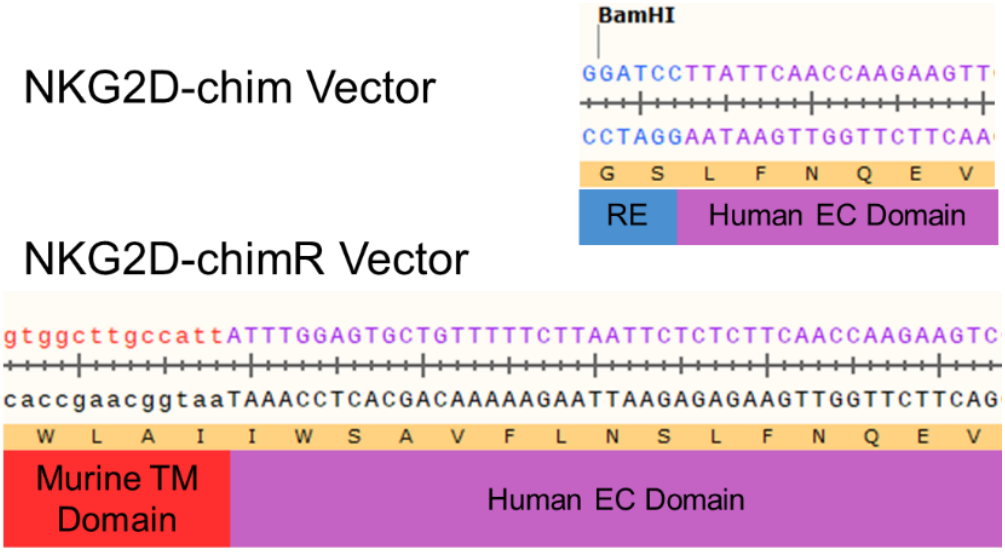

Figure 14. The protein sequence for the NKG2D-chim receptor is compared with the NKG2D-chimR receptor. The human extracellular domain in the NKG2D-chimR vector has been codon optimized for murine expression, but by comparing the protein sequences we see that the NKG2D-chim vector is missing eight amino acids (IWSAVFLN) from the human extracellular domain.

293T cell NKG2D-chimR binding assay

293T cells transiently expressing NKG2D-chimR vector were tested for human NKG2D binding using human NKG2D ligands, including anti-human NKG2D antibodies and our BiKE. Both treatments have an increase in mean fluorescence intensity (MFI) when compared to the isotype control (Fig. 15). These results indicate that our newly designed NKG2D-chimR vector can bind human NKG2D ligands.

Figure 15. 293T Cells NKG2D-chimR Binding Assay

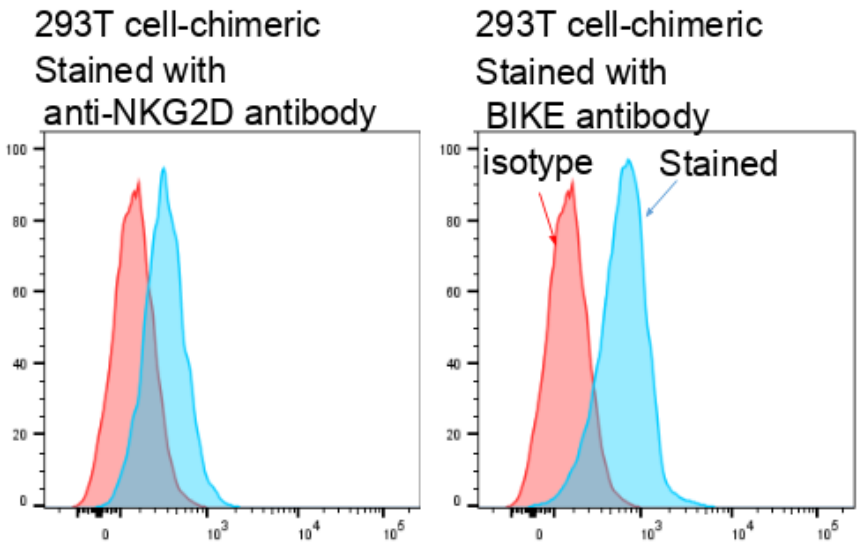

Figure 15. NKG2D-chimR transfected 293T cells stained for human NKG2D using anti-human NKG2D antibodies and fluorescent BiKE antibodies. Both treatments show a shift in fluorescence, indicating NKG2D-chimR expression and binding with human NKG2D ligands.

Discussion

Multiple myeloma is an incurable hematological malignancy with over 30,000 diagnoses per year (5). With limited treatment options, many MM patients face ongoing chemotherapy treatments and drug side effects that affect their quality of life. Recently, the field of immunotherapy has created opportunities to redirect the immune system against MM, and our lab has capitalized on this by creating a MM-specific BiKE. BiKE is a bispecific antibody targeting both CS1, a receptor highly expressed on MM tumor cells, and NKG2D, and activating receptor on NK, NKT, and T cells. While our lab has shown BiKE to be effective *in vitro*, our *in vivo* model does not accurately model a BiKE drug response, and this preclinical data is critical to begin testing BiKE for the FDA. Thus, an immunocompetent mouse model must be created to test BiKE *in vivo*.

The purpose of this study was to make and test a chimeric NKG2D receptor functionality to make a mouse model to test BiKE efficacy in mice. We hypothesized that by creating a chimeric NKG2D protein, we could create a mouse model that expresses chimeric NKG2D on NK, NKT, and T cells naturally in the murine immune system, respond to human NKG2D ligands, and activate NK cells through IFNγ release and cell cytotoxicity. Specifically, we wanted to create an immunocompetent mouse model that could respond to our anti-NKG2D and anti-CS1 BiKE protein. This mouse model is critical to produce preclinical *in vivo* data about BiKE to lend support for BiKE clinical trials and could also be used to test other aspects of human NKG2D in a mouse model.

To test our construct, we expressed our chimeric protein in LNK and primary murine spleen NK cells to test NKG2D functionality through cell cytotoxicity, a key action of NK cells

to lyse target cells. While our LNK cytotoxicity assay did not show statistically significant killing of MM cells, our primary murine NK cell assay did show significant MM cell lysis. Although we demonstrated that primary murine NK cells elicit a tumor killing response, there was concern that the response may not be as strong as human NK cells incubated with BiKE. While we later found that the chimeric protein used in these experiments was faulty, the positive results in the primary murine NK cells bode well for our continuing experiments with our redesigned protein. Now that we have redesigned the chimeric protein we are optimistic that it may elicit a stronger tumor killing response and prove a promising candidate for a murine mouse model.

While our LNK chimeric protein cytotoxicity assay was unsuccessful, the same experiment in primary murine NK cells lended statistically significant results. We believe this may be due to functional differences in the LNK cell line. While the LNK cell line is immortalized, simple to culture, and is easier to transduce than primary NK cells, there is limited data on the functionality of the LNK cell line in the literature (46,47). So, we began using primary spleen murine NK cells instead to better model the functionality of murine NK cells. However, this has presented challenges because primary cells are not immortalized and they are difficult to transduce (47). Ultimately, we decided it was better to accurately model the functionality of murine NK cells, perhaps explaining the different results in the cytotoxicity assays.

When redesigning our chimeric protein, we found that our NKG2D-chim vector contained an extraneous murine NKG2D-S extracellular domain and a human NKG2D extracellular domain deletion. The DNA sequence was then corrected and to improve

functionality the restriction enzymes cut sites were removed. Currently, the sequence contains the murine NKG2D-S intracellular and transmembrane domains and the human NKG2D ectodomain. As a result, the redesigned protein should bind human NKG2D ligands, such as BiKE, with the human ectodomain while also associating with both murine DAP10 and DAP12 using the murine NKG2D-S transmembrane and intracellular domains. Our results also support previous literature indicating that species-specific NKG2D association with DAP10 occurs in the transmembrane domain (32). By utilizing the murine NKG2D-S transmembrane domain in our chimeric protein, we were able to induce cell cytotoxicity using human ligands, indicating that the transmembrane domain, not the extracellular domain, is important for this signaling process.

Finally, we tested the ability of BiKE and anti-human NKG2D antibodies to bind to our chimeric receptor. To elicit an immune response, the chimeric construct must bind human NKG2D ligands, so we use flow cytometry and fluorescent ligands to examine this binding. In a previous pilot study, our lab showed that primary murine NK cells expressing the NKG2D-chim vector did not bind human NKG2D ligands (data not shown). However, when we transiently transformed 293T cells with the redesigned NKG2D-chimR vector, we saw the expression of NKG2D and binding of both BiKE and anti-human NKG2D antibodies. These results indicate that our chimeric protein can bind human ligands and also indicate that our chimeric protein may better respond to ligands to produce both cell cytotoxicity and IFNγ release. Looking forward, we are optimistic that the new chimeric NKG2D protein will provide stronger cell cytotoxicity and IFNγ release.

One limitation in this study is our initial NKG2D-chim trials in LNK cells did not lend statistically significant data showing NKG2D activation. However, using the primary murine NK cells we were able to overcome this limitation. In addition, we had a small number of trials and cell numbers for our experiments due to the toxicity of the NK cell transduction process. Literature shows that lentiviral transduction is the most effective means of expressing foreign DNA in NK cells, but these methods are still only 15% effective, decreasing the number of cells we can use for our experiments (47). Finally, the misdesigned chimeric protein used in the cell cytotoxicity assay prevents us from further interpreting negative results. However, the chimeric protein has been redesigned so moving forward with the project we will be better able to interpret our results. Despite these limitations, positive results from binding assays in transient 293T cells indicate the validity of our methods and the potential for this project's future success.

In conclusion, our chimeric NKG2D protein has been shown to bind human NKG2D ligands and initiate a cytotoxic response. While more testing must be conducted on our NKG2D-chimR vector, we are optimistic that this data will support the creation of a chimeric NKG2D knock-in mouse model. This murine model will express NKG2D in NK, NKT, and T cells, creating a more accurate model of the activating effects of BiKE *in vitro* than NK cells alone. In addition, this model will allow us to stop injecting human NK cells into mice to test BiKE, reducing the amount of error we are introducing into our modeling system. Due to BiKE's modular design, other researchers may continue to use an anti-NKG2D BiKE therapy targeting different cancer targets for other cancer therapies. If so, creating this mouse model will serve to expedite other BiKE clinical and translational research projects. To the best of our knowledge, there is no other mouse model available expressing a chimeric NKG2D

receptor. Finally, in addition to helping test BiKE, the mouse model may serve as a better system to study human NKG2D cell responses *in vitro*, fulfilling a need in the greater scientific community. By creating this model, we are one step closer to providing BiKE therapy to multiple myeloma cancer patients.

Future Directions

Since we have confirmed our new NKG2D-chimR receptor can bind BiKE in 293T cells, our next step would be to create viral NKG2D-chimR particles and transduce murine NK cells. Once we see GFP expression in the cells, we would retest the binding of BiKE to the transduced receptor. Given our previous results with 293T cell expression, we expect that our construct will still bind in murine NK cells. If, however, it does not bind BiKE, this would point towards post-transcriptional mechanisms preventing expression of the chimeric receptor on the surface of the cell. If this occurred, we would investigate mRNA levels of our chimeric receptor, glycosylation of the protein, and protein trafficking through the ER.

Next, we would test the redesigned chimeric receptor's ability to induce a cytotoxic response to reject tumor cells. In this experiment, we would utilize human NK cells as a positive control, our empty vector murine NK cells as a negative control, and murine NK cells expressing our chimeric receptor as our test cell line. These cell lines would be seeded with multiple myeloma tumor cells (MM1.s), and there would be both a control group receiving our control BiKE and a group receiving a BiKE treatment. The assay would be read by the BD LSRII flow cytometer, as described earlier in the methods. We would look for statistically significant tumor clearance in the chimeric cell line receiving a BiKE treatment, similar to the tumor cell clearance of human NK cells with a BiKE treatment. If this experiment does not show tumor clearance, we would investigate the downstream signaling pathway of NKG2D in mice to see which parts of the signaling pathway are disrupted. This would include testing the downstream phosphorylation of murine DAP10 and the PI3 kinase. If these signaling pathways are not activated, we may have to redesign the transmembrane domain of the

chimeric protein to ensure activation of these pathways. If the cytotoxicity assay works, then we will proceed to test IFNγ release.

To test IFNγ levels, we would perform a crosslinking assay to induce IFNγ production. Here again we would utilize human NK cells, murine NK cells with an empty vector, and murine NK cells expressing the redesigned chimeric NKG2D receptor. Each cell line would be incubated with anti-human NKG2D antibodies, and then subsequently incubated with anti-human mouse antibodies. Using a golgi plug to block the release of the IFNγ to medium, we would determine the IFNγ expression levels using flow cytometry and staining for intracellular IFNγ. We can also perform an ELISA experiment using the supernatant from the cytotoxicity assay cultures to determine IFNγ levels. If these experiments are not showing the release of IFNγ, then we will examine the downstream signaling of murine DAP12, including the phosphorylation of DAP12, phosphorylation of Syk kinase, and the production of NFκB. We also would check for coimmunoprecipitation of Syk kinase with DAP12 and our NKG2D-chimR receptor. If these signaling pathways are not being activated, we may need to redesign the transmembrane domain of our chimeric NKG2D protein.

If found that neither the cytotoxicity assays or the IFNγ assays are showing NKG2D activation, then we will consider co-transducing human DAP10 into murine NK cells with the full length human NKG2D receptor. We also will investigate the processing of our chimeric NKG2D receptor, including intracellular trafficking and glycosylation. However, if our assays show that our NKG2D chimeric receptor is activating murine NK cells, we will proceed to make a heterozygous knock-in mouse model to test BiKE *in vivo* using the OSU Genetically Engineered Mouse Model Core. We will repeat the same mouse experiment as described in

the intro, except we will not have to inject human NK cells into the mouse. This trial will generate preclinical data for BiKE and the data collected may even help move our BiKE treatment to clinical trials.

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